

Characterization of a spontaneously transformed chicken mononuclear cell line[☆]

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Abstract

We describe the characterization of a spontaneously transformed chicken monocytic cell line that developed as a single colony of cells in a heterophil culture that was inadvertently left in the incubator over a period of 25 days. These cells, hitherto named HTC, grow efficiently at both 37 or 41 °C in culture medium containing either 5% FBS or 2% chicken serum. The HTC cells are acid phosphatase positive, show expressions of both class I and class II major histocompatibility complex (MHC), CD44, K1, and K55 cell surface antigens, and engulf latex beads, produce nitrite and interleukin-6 on stimulation with bacterial lipopolysaccharide (LPS). Treatment with phorbol myristate acetate (PMA) induces respiratory burst in HTC cells and the secretion of matrix metalloproteinase (MMP) into culture medium. Using gene-specific primers and reverse transcriptase-polymerase chain reaction (RT-PCR), the presence of mRNA transcripts for interferon- γ (IFN- γ), interleukin-1 (IL-1), interleukin-6 (IL-6), nitric oxide synthase (NOS), matrix metalloproteinase-2 (MMP-2), and transforming growth factor- β (TGF- β) were detected. Lipopolysaccharide (LPS) treatment of HTC cells modulated IL-1, IL-6, IFN- γ , NOS mRNA levels as detected by RT-PCR analyses. Using different avian tumor virus gene-specific primers and PCR, the HTC cells were positive for the presence of avian leukosis virus (ALV) and Marek's disease virus (MDV) but negative for reticuloendothelial virus (REV), chicken infectious anemia virus (CIAV), and herpes virus of turkeys (HVT). The production of ALV antigens by HTC cells was further confirmed using p27 gag protein ELISA. Collectively, these results show that the HTC cells belong to myeloid/macrophage lineage and were likely transformed by ALV and MDV but retain many interesting and useful biological activities. Published by Elsevier B.V.

Keywords: HTC cells; Transformed macrophage; Chicken

Abbreviations: ALV, avian leukosis virus; CE, capillary electrophoresis; CIAV, chicken infectious anemia virus; CS, chicken serum; DAPI, 4',6-diamidino-2-phenylindole; DCF-DA, 2',7'-dichlorofluorescein diacetate; ELISA, enzyme-linked immunoabsorbent assay; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; HVT, herpesvirus of turkeys; IFN, interferon; IL, interleukin; iNOS, inducible nitric oxide synthase; LIF, laser-induced fluorescence; LPS, lipopolysaccharide; MDV, Marek's disease virus; MMP, matrix metalloproteinase; MTT, (3-(4,5 dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide; REV, reticuloendothelial virus; PMA, phorbol myristate acetate; RT-PCR, reverse transcriptase-polymerase chain reaction; SPF SCWL, specific pathogen free single comb white leghorn; TGF- β , transforming growth factor- β

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1. Introduction

Transformed cells provide useful models to study the functional physiology of their normal counter parts because of their abilities to undergo numerous cell divisions. These cells can occur both in vivo or in culture because of natural mutations or through neoplastic transformation induced by viruses, chemicals, and genetic manipulation. The transformed cells have not only been useful in the understanding of basic cellular physiology but also have provided insight into the mechanisms of neoplastic transformation, discovery of oncogenes, growth factors and gene regulatory mechanisms. Additionally, the transformed cells have also been used for cultivating compatible viruses (Kawaguchi et al., 1987) and bioassays of growth factors, and cytokines (Mire-Sluis and Thorpe, 1999). However, it is useful to know the identity of transformed cells for such studies which is often known from the tissues of their origin, otherwise, it may be necessary to identify and characterize these cells using specific morphological and functional markers.

In the course of culturing peripheral blood heterophil–granulocytes from broiler chickens, we observed a single colony of cells in a culture flask which was inadvertently left in the incubator over a period of 25 days during which time the heterophils had died. To understand the identity, we expanded these cells in RPMI-1640 medium containing 10% FBS and examined for the presence of granulocyte features associated with heterophils using conventional and FITC staining procedures (Rath et al., 1998). Because these cells were devoid of granulocytic features which included bilobed nuclei and cytoplasmic granules, we further examined them using different morphological and molecular markers, and functional assays. These cells, operationally named as HTC have been grown over 75 passages in culture medium containing either 2% CS or 5% FBS and show many characteristics attributed to cells of a myeloid/macrophage lineage.

2. Materials and methods

2.1. Reagents and media

All tissue culture media and reagents were obtained from commercial sources unless otherwise specified.

These include tissue culture medium RPMI-1640 (VWR Scientific Products, Pittsburgh, PA), FBS (HyClone, Logan, UT), 2',7'-dichlorofluorescein diacetate (DCF-DA) and carboxylate-modified 0.2 and 1 μ m fluosphere latex beads (Molecular Probe, Eugene, OR), recombinant human IL-6 (Roche Diagnostic Corporation, Indianapolis, IN), Qiagen RNAeasy RNA extraction kit (Qiagen, Chatsworth, CA), Mark 12TM broad range protein standard, and oligonucleotide primers (Invitrogen, Carlsbad, CA), RETROscriptTM two step RT-PCR kit (Ambion, Austin, TX), 1 kb base pair ladder (Promega Corporation, Madison, WI), chicken cell surface antigen-specific monoclonal antibodies (Southern Biotechnology Associates, Birmingham, AL), μ SIL DNA capillaries with an internal diameter of 75 μ m (Agilent Technologies, San Jose, CA), DNA intercalating dye Enhance (Beckman, CA), and ProFlores ELISA kit (KPL, Gaithersburg, MD). All other chemicals, reagents, cell culture additives, including chicken serum, PMA, DNA gel buffer, *Salmonella typhimurium* LPS were purchased from Sigma Chemical (St. Louis, MO). The leukocyte-common antigen-specific monoclonal antibodies K1, K55 (Chung et al., 1991; Cloud et al., 1992), Bu-1a, and IgM were kindly provided by H. Lillehoj (USDA/ARS, Beltsville, MD).

2.2. Cells

The HTC cells were grown in RPMI-1640 medium containing 1 \times concentration of antibiotic–antimycotic solution, gentamicin (10 μ g/ml), 1 mM Na-pyruvate, 2 mM glutamine, 50 μ M β -mercaptoethanol, and 5% FBS or 2% chicken serum (v/v). Although these cells could grow equally well both at 37 or 41 $^{\circ}$ C, most studies were done with cells that were grown in medium containing 2% CS at 37 $^{\circ}$ C. In low densities the HTC cells grew as loose monolayers but tended to detach from the bottom as growth progressed, formed aggregates and continued to grow in suspension. The cells were passaged at a ratio of 1:10 every 5 days with fresh medium. For some experiments the cells were grown on sterile cover slips. For most analyses, cells that had grown beyond 75 passages were used. B9 hybridoma cells (obtained from L. Aarden, The Netherlands Red Cross Laboratory, The Netherlands) were maintained in RPMI 1640

medium containing 10% FBS and 10 U rhIL-6/ml medium and used on the 4th day of culture for IL-6 assays (Xie et al., 2002). For MDV and HVT infections, SPF-SCWL chicken embryo fibroblasts were used. MDV strains RB1B and HVT (FC-126) were obtained from Dr. J.K. Rosenberger, University of Delaware. CIAV-infected MSB-1 cells (Akiyama and Kato, 1974) were provided by Dr. L. Newberry, University of Arkansas, ALV-transformed B cell line DT40 (Baba et al., 1985), and RP9 (Sharma and Okazaki, 1981) were obtained from Dr. R. Hrdlickova, University of Texas at Austin, and Dr. H.D. Hunt, USDA-ADOL, East Lansing, MI, respectively. REV-transformed B cell line CU60 and T cell line CU91 (Pratt et al., 1992) were provided by Dr. K.A. Schat, Cornell University, Ithaca, NY. The RB1B-transformed T cell line UD14 was established from a RB1B-induced tumor (Parcells et al., 1995). Monocytic line UA05 was established from a commercial broiler myeloid spleen tumor (Parcells, unpublished). Macrophage cell lines MQ-NCSU (Qureshi et al., 1990) and HD11 (Beug et al., 1979) were provided by Dr. M. Qureshi, North Carolina State University and Dr. D. Barnes, University of Arkansas. Uninfected and infected CEF were grown in medium 199 containing 3% FBS and all other B cell, T cell, and macrophage lines were grown in RPMI-1640 medium containing 20% FBS, and $1 \times$ antibiotic–antimycotic solution.

2.3. Giemsa staining of cells

The cells were stained with Wright's Giemsa (Lucas and Jamroz, 1961). Cells grown on cover slips were fixed using 4% *p*-formaldehyde and washed with water before staining. The single cell suspensions of HTC cells were attached to the slides using a Cytopro centrifuge (Wescor Inc., Logan, UT) before fixation and staining as above.

2.4. Cell growth

The growth rates of HTC cells were determined using an MTT reduction assay (Xie et al., 2002). Single cell suspensions of HTC cells were seeded at a concentration of 10^5 cells/ml of medium containing 2% CS in 24 well culture plates at volumes of 0.5 ml per well and cultured at 37 and 41 °C. At different

times after plating, the cells were incubated with MTT at a concentration of 0.5 mg/ml for 3 h, centrifuged at $400 \times g$ to remove supernatant, dissolved in 200 μ l of lysis buffer (Hansen et al., 1989), transferred to a 96-well plate and the OD of reduced formazan read at 570 nm using a microplate reader. Each time point consisted of triplicate wells of culture. The percent changes in OD were calculated relative to the OD of cells at the onset of culture (100%) and were used to represent the changes in cell density.

2.5. Acid phosphatase staining

The cells were seeded as above at a concentration of 10^5 cells/ml, on sterile microscope cover slips placed in 12 well culture plates and grown for 3 days, then fixed with 4% *p*-formaldehyde for 3 h, washed briefly once with PBS and twice with water 5 min each. The cells were stained for total and tartarate resistant acid phosphatase using a leukocyte acid phosphatase kit (Sigma Chemical, St. Louis, MO) according to the manufacturer's instructions.

2.6. Flow cytometric analyses

To identify the immunophenotype of the HTC cells, samples were stained with mouse monoclonal antibodies raised against known lymphocyte and macrophage/thrombocyte surface antigens and counter stained with anti-mouse secondary antibody conjugated to phycoerythrine (PE), and subjected to flow cytometric analyses essentially as described by Parcells et al. (1995). The immunophenotype of these cells were compared with other cell lines of known identities. These cell lines included B cells, DT40 (Baba et al., 1985), RP9 (Sharma and Okazaki, 1981), and CU60 (Pratt et al., 1992), T cells, CU91 (Pratt et al., 1992), and UD14 (Parcells et al., 1995, 1999), and macrophage lines, HD11 (Beug et al., 1979), and MQ-NCSU (Qureshi et al., 1990). Briefly, the HTC and other control chicken cells were suspended in PBS containing 3% goat serum, 1% bovine serum albumin, and 0.1% sodium azide at a concentration of 1×10^5 cells/ml, were incubated with different monoclonal antibodies (Table 1) for 30 min on ice, washed twice with PBS containing 0.1% NaN_3 , and the wash supernatant was removed by centrifugation at $200 \times g$. The cells

Table 1

Comparative flow cytometric profiles of different transformed chicken cell lines including HTC cells^a

Antigen-specificity	B cells			T cells		Macrophage		
	DT40	RP9	CU60	CU91	UD14	MQ-NCSU	HD11	HTC
CD3	–	–	–	+	++	–	–	–
CD4	–	–	–	±	+++	–	–	–
CD8	–	–	–	–	–	–	–	–
CD8b	–	–	–	–	–	–	–	–
TCR1	–	–	–	–	–	–	–	–
TCR2	–	–	–	–	++	–	–	–
TCR3	–	–	–	±	+	–	–	–
CD44	ND	ND	ND	++++	+++	+++	+++	+++
Bu-1a	40	++	++	–	–	–	–	–
IgM	++	+	++	–	–	–	–	–
K55	++	+	++	++	++	+++	+++	++
K1	–	±	–	–	–	±	+	+
MHC-I	+	+++	+	+++	+++	+++	+++	+++
MHC-II	+	++++	+	++++	+++	+	++	+

^a 0–≤2% (–), 3–5% (±), ≤20% (+), 20–50% (++) , 50–100% (+++), 100% cells with intensity of $\geq 10^3$ fluorescence units (++++).

were incubated with phycoerythrin conjugated goat anti-mouse IgG antibody for 30 min on ice, and after washing the cells as above they were resuspended in PBS and analyzed with a FACSort flow cytometer equipped with Cellquest software (Becton Dickinson, San Jose, CA). Based upon % population of cells showing fluorescence, analyzed from 10,000 cells, they were arbitrarily assigned as 0–≤2% (–, no fluorescence), 3–5% (±), 6–20% (+), 20–50% (++) , 50–100% (+++), and 100% cells with intensity of $\geq 10^3$ fluorescence units (++++).

2.7. Phagocytosis assay

HTC cells adjusted to a concentration of 10^6 cells/ml were incubated with sonicated and dispersed carboxylate-modified fluosphere latex beads (0.2 or 1 μ m diameter) suspended in sterile PBS to a final concentration of 0.01 or 0.1 mg/ml, and the cells were harvested at 0, 2, and 8 h post-treatment. Immediately after addition or at different times after the addition of beads, 0.2 ml of cell suspension was layered over 1 ml of chicken serum in a microcentrifuge tube, and centrifuged for 20 min at $450 \times g$ and the cell pellet was resuspended in 0.2 ml of PBS, subjected to cyto-centrifugation and staining with Wright's Giemsa or DAPI for fluorescence. Cells were examined and photographed using an OlympusBX50 fluorescence microscope.

2.8. Assay of nitrite, IL-6, and MMP in HTC-cell conditioned media after treatment with ionomycin, LPS, PMA, and zymosan

The cells were suspended at a concentration of 2×10^6 cells/ml RPMI 1640 medium containing 2% chicken serum as above and cultured at volumes of 250 μ l cells/well in 24-well tissue culture plates. LPS was added at concentrations of 0, 0.1, 1, and 10 μ g/ml for 24 h after which the cells were centrifuged at $340 \times g$ for 5 min. The supernatant medium was used to measure IL-6 according to earlier procedures using the B9 cell bioassay (Helle et al., 1988; Rath et al., 1998) and nitrite using Griess reagent according to Ding et al. (1988). Human rIL-6 and sodium nitrite were used as standards, respectively. Each of these assays was repeated twice on different days and the samples were run in triplicate. Wells containing identically treated culture medium without cells were used to test for background associated activities. Relative changes in the concentrations of both nitrite and IL-6 were calculated with respect to the levels in the untreated wells.

Based on preliminary evidence which showed the ability of HTC cells to produce matrix metalloproteinase (MMP) activities, we compared the effects of ionomycin, LPS, PMA (final concentration 1 μ g/ml each), and zymosan (10 μ g/ml) to induce the production of MMP at 24 h. MMP activity of the conditioned media was determined using gelatin substrate

zymography (Rath et al., 1998, 2001). Briefly, aliquots of conditioned medium from each culture was pre-diluted in non-reducing sample buffer (2×) and applied to 10% SDS-PAGE (acrylamide:bis, 19:1) impregnated with 0.1% gelatin and electrophoresed at 100 V constant for 3 h. Mark 12™ broad range protein standard was used as a molecular weight reference. The gels were washed with 2.5% Triton X-100 twice, 15 min each followed by a brief rinse in a collagenase buffer (50 mM Tris HCl, 5 mM CaCl₂, 100 mM NaCl, 0.02% NaN₃, pH 7.4), and incubated at 37 °C overnight in the same buffer. After a standard procedure of staining with Coomassie blue and destaining, the gels were air-dried in cellulose acetate membranes, and photographed. The gelatinolytic bands, represented the MMP activities of the samples. Approximate molecular size of gelatinolytic bands were determined based on molecular marker standards using a Geldoc system (BioRad, Hercules, CA).

2.9. Respiratory burst activity measurement using PMA-induced DCF-DA oxidation

The respiratory burst activities of HTC cells were measured using DCF-DA oxidation (Rath et al., 1998). HTC cells (10⁶ cells/100 µl) were suspended and cultured in phenol red free RPMI-1640 medium containing 2% CS and 20 µg of DCF-DA/ml in 96 wells and added with 0, 10, 100, or 1000 ng PMA/ml for a period of 60 min. The changes in the fluorescence (AFU) due to the DCF-DA oxidation products by the reactive oxygen species was monitored immediately after the addition of PMA and at 60 min using a Cytofluor 4400 fluorescence plate reader (Millipore, Bedford, MA) following incubation at 37 °C. The assays were run in triplicate and repeated twice on different days using cells from different passages. The changes in fluorescence were also monitored morphologically using cover slip-cultured HTC cells which were incubated with DCF-DA as above for 10 min, then treated with 100 ng PMA/ml of medium, and photographed within 10 min using a fluorescent microscope.

2.10. RT-PCR analyses for IFN-γ, IL-6, IL-1, NOS, MMP-2, TGF-β4, gene expression

Replicate cultures of HTC cells were grown in 12-well plates at the concentration of 2 × 10⁶ cells/ml/well

for 18 h before the addition of LPS at a concentration of 10 µg/ml to one set of culture for 6 h and immediately before the harvest (0 h) to the other set for the extraction of RNA. After removing the medium and washing the cells once with sterile PBS, the RNA was purified using an RNAeasy kit (Qiagen, Chatsworth, CA) according to the manufacturer's instruction. The RNA purity was determined using 1% agarose gel electrophoresis and ethidium bromide staining, yield using OD at 260 nm (Ausubel et al., 1999). The RNA was frozen in aliquots at −80 °C for later uses. Two hundred nanograms of total RNA from duplicate wells of each treatment group was reverse transcribed using RETROscript, 2-step RT-PCR kit where the first strand was synthesized using random decamers (Ambion, Austin, TX). A master mix containing reaction buffer, dNTPs, Taq polymerase, and 1 µl cDNA/20 µl reaction mixture was distributed into different PCR tubes to which each of forward and reverse primers (final concentration 0.25 µM each) corresponding to different individual genes (Table 2) were added and subjected to PCR amplification using primer sets designed against each of β-actin, IFN-γ, IL-1β, IL-6, iNOS, TGF-β4, and MMP-2. Based on preliminary optimization experiments, the reactions were run for 22 cycles for β-actin mRNA and 35 cycles for the rest. The primer pairs and the reaction conditions used for these chicken specific genes were adapted from published literature (Table 2). Except for the annealing temperature that was maintained at 60 °C for all primers, the rest of the conditions included 94 °C for denaturing followed by extension at 72 °C for 2 min. At the end, the PCR products were diluted 10-fold with water and analyzed using a PACE 5500 capillary electrophoresis (CE) system equipped with a laser-induced fluorescence (LIF) detector using a procedure developed by Richards and Poch (2002). The capillary was filled with a DNA gel buffer diluted to 75% concentration with double distilled water that contained a DNA intercalating dye ENhance (20 µl/ml). The samples were injected electrokinetically at 3 kV in the cathode end of the capillary and separated at a constant 7 kV for 2.75 min in a µSIL DNA capillary with an internal diameter of 75 µm (Agilent Technology, San Jose, CA). In between separations the capillary was rinsed for 1 min with methanol followed by 1 min rinse with a fresh gel buffer. The total separating distance from the inlet to the detector

Table 2
PCR primers for mRNA targets

Test genes	Forward primer	Reverse primer	Amplicon size (bp)	References
β -Actin ^a	5'-CCATGAACTACC-TTCAACTCCA-3'	5'-GATTCATCGTACTCCT-GCTTGCT-3'	273	Kost et al. (1983)
IFN- γ	5'-GCTGACGGTGGA-CCTATTATT-3'	5'-TGGATTCTCAAGTCG-TTCATCG-3'	247	Digby and Lowenthal (1995), Leshchinsky and Klasing (2001)
IL-1 β	5'-GGCTCAACA-TTGCCTGTAC-3'	5'-CCCCTTAGCTTG-TAGGTGGC-3'	350	Leshchinsky and Klasing (2001)
IL-6	5'-GCTCGCCGGC-TTCGA-3'	5'-GGTAGGTCTGAAAG-GCGAACAG-3'	191 (non-coding sequence), 71 (coding sequence)	Schneider et al. (2001), Kaiser et al. (2001)
iNOS	5'-AGGCCAAACA-TCCTGGAGGTC-3'	5'-TCATAGAGAC-GCTGCTGCCAG-3'	371	Djeraba et al. (2002)
TGF- β 4	5'-ACCTCGACACC-GACTACTGCT-3'	5'-CTGCACTTGCA-GGCACGGAC-3'	340	Laurent et al. (2001)
MMP-2	5'-GCAAGAAGGGC-ATCACCGAAGAA-3'	5'-ACGACGAATGCGC-GATAACAAAAT-3'	578	Aimes et al. (1994), Nie et al. (1998)

^a Designed from sequences published by Kost et al. (1983).

was 7.5 cm. The base pair size estimates of the PCR amplicons were determined using a standard curve generated using a standard 1 kb DNA ladder. The relative changes in the expression of different genes were calculated with respect to β -actin by dividing the peak area density of the different amplicons with the peak density of β -actin for both 0 and 6 h samples. The average changes were based on estimation from duplicate samples. For visual documentation of the amplicons, 2–8 μ l of PCR samples were separated on a 1.5% agarose gel, stained with ethidium bromide using a standard procedure, and photographed. The % CV for molecular size estimates of different amplicons was within 1–5% of range using the CE-LIF method.

2.11. Determination for the presence of viral genome by PCR

DNA purified from chicken embryo fibroblasts (CEF) derived from SPF-SCWL embryonated eggs and different lymphocytic and monocytic lines positive for different viral genes were used as controls along with HTC cells. The DNA from these cells was extracted using standard methods (Sambrook et al., 1989) and amplified with different primers specific for avian leukosis virus (ALV), chicken infectious anemia virus (CIAV), herpes virus of turkeys (HVT), three pairs of primers for Marek's disease virus (MDV), and reticuloendothelial virus (REV) (Table 3). The PCR

assays were performed in a total volume of 50 ml consisting of 100 ng of DNA, Taq supermixII, and 0.1 nM concentration of each of the primers for respective viruses (Table 3). The reaction mixtures were analyzed for specific amplicons using 1.2% agarose gel electrophoresis (Ausubel et al., 1999), stained with ethidium bromide, and photographed. Approximate size of the amplicons were determined using molecular size markers.

2.12. P27 enzyme-linked immunoabsorbent assay (ELISA)

To find whether the cells were shedding ALV antigens, we assayed the conditioned medium (1000 \times g supernatant filtered through a 0.22 μ m filter) of freshly grown HTC cells ($\sim 1.2 \times 10^6$ cells/ml) and pelleted cell extract prepared by sonication (6×10^6 cells/ml fresh RPMI-1640) using a ProFloek ELISA kit (KPL, Gaithersburg, MD) that detects an ALV group specific antigen (GAG) P27. To determine whether the ALV belonged to the subgroup J that is prevalent in many commercial broiler flocks (Payne, 1998), we inoculated secondary chicken embryo fibroblasts (CEF) with 0.22 μ m filtered cell culture supernatant from replicate HTC cultures; UA05 cells were used as ALV-J positive control (Parcells et al., unpublished), and culture medium alone inoculated CEF as negative control. Indirect immunofluorescence staining of the

Table 3
PCR primers for viral genes

Virus	Forward primer	Reverse primer	Amplico <i>n</i> size (bp)	Reference
ALV ^a	5'-GTATGATCGTGCC- TTATTAGGAAGG-3'	5'-ACCCAGGTGCACACCAATGTGG-3'	152	This report ^a
CIAV ^b	5'-CTAAGATCTGCAACTGCGGA-3'	5'-CCTTGGGAAGCGGATAGTCAT-3'	420	NVSL ^b
HVT	5'-TAGGAATTCATGGGTAT- GTATGGTTGCATGA-3'	5'-TCTGGATCCTTAGGCGA- CCCTATTCCAATAC-3'	1303	This report ^c
MDV-US2	5'-GTACCACGAACTCCAGTTC-3'	5'-CTTCCGCGTTATCGGTAC-3'	534	Dienglewicz and Parcells (1999) ^d
MDV-US3	5'-CTCAGGGTTAGACTTCTAAC-3'	5'-GACGGGTGTTTACATATGAG-3'	1015	This report ^c
MDV (ICP27)	5'-GCAAGAAGGGCATCACC- GAAGAA-3'	5'-ACGACGAATGCGCGATAAC- AAAAT-3'	670	Parcells et al. (2001)
REV	5'-CATACTGGAGCCAATGGTT-3'	5'-AATGTTGTACCGAAGTACT-3'	292	Silva et al. (1997) ^f

^a Exogenous ALV primer sets (Accession # Z46390) designed by Dr. R. Okimoto, University of Arkansas detects all exogenous ALVs (not subgroup E).

^b Taylor SP, National Veterinary Services Laboratory (NVSL), USDA, ARS, Ames, IA.

^c Virion-host shut-off (UL41) gene of HVT, Accession # AF291866, italic sequences are restriction sites added for cloning of this product, from published sequence by Afonso et al. (2001).

^d Dienglewicz and Parcells (1999).

^e This report, primers designed from published sequence of MDV-US3 genome by Tulman et al. (2000).

^f Reticuloendotheliosis virus (REV) LTR-specific primer, Silva et al. (1997).

CEF was done 7 days post-inoculation using mouse monoclonal antibody specific for gp85 (Qin et al., 2001), a surface component for the *env* gene product of the ALV-J subgroup. Antibody binding was detected using goat anti-mouse antibody-FITC conjugate (1:100 dilution) and by direct observation using a fluorescence microscope.

2.13. Statistical analyses

Data were expressed as means \pm S.E. and analyzed by Student's *t*-test wherever necessary.

3. Results

3.1. Cells and growth

The HTC cells grew as loosely adherent cells in low density but often as aggregates in suspension at high density. The presence of 2% CS alone was sufficient to support the cell growth. The cells often showed cytoplasmic projections and a part of cytoplasm appeared rarefied with mature cells containing cytoplasmic vacuoles (Fig. 1A). The HTC cells were positive for acid phosphatase including the tartarate resistant acid

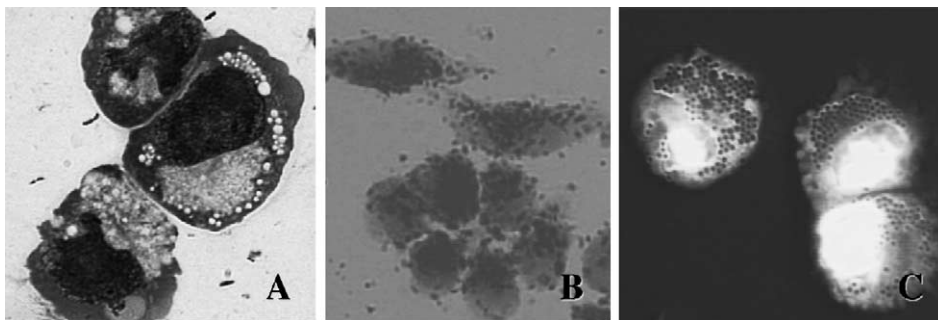


Fig. 1. (A) Giemsa staining of cytocentrifuged HTC cells; (B) acid phosphatase staining of HTC cells cultured on a cover slip; (C) phagocytosis of latex fluospheres (10 μ g/ml) by HTC cells at 8 h of incubation.

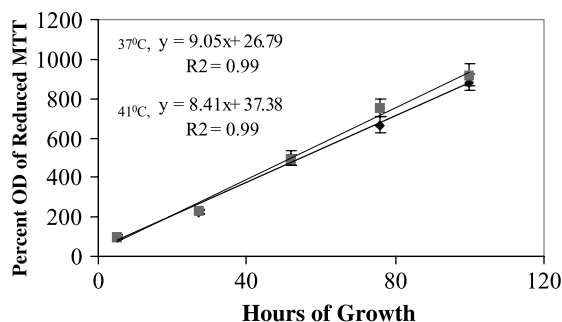


Fig. 2. MTT reduction by HTC cells growing at 37 and 41 °C at different times of growth.

phosphatase (Fig. 1B). These cells grew at a constant rate for at least up to 100 h of culture in medium containing 2% CS as evident by MTT reduction assay (Fig. 2). The growth rates of HTC cells were comparable at both 37 and 41 °C.

3.2. Flow cytometric analyses

The immunophenotyping of the HTC cells using a panel of mouse anti-chicken monoclonal antibodies showed these cells to be positive for CD44, K1, K55, MHC class I, and MHC class II antigens (Table 1). A similar pattern of staining was seen in HD11 and MQ-NCSU cells both of which belong to the macrophage class of cells.

3.3. Phagocytosis assay

Phagocytosis of latex beads by the HTC cells is shown in Fig. 1C. Whereas the cells showed no detectable ingested latex beads during first 10–15 min, the beads were detected in the cytoplasm after 2 h and significant accumulation occurred at 8–24 h to the extent that by 48 h many cells exhibited impeded cytokinesis due to the accumulating latex beads in the cytoplasmic vacuoles (not shown).

3.4. Effects of LPS and PMA on HTC cells

LPS stimulated the production of both nitrite and IL-6 (Fig. 3). PMA-induced respiratory burst in HTC cells evident by an increase in DCF-DA fluorescence (Fig. 4A and B) which was not induced by LPS (not shown). Similarly, PMA treatment induced significant

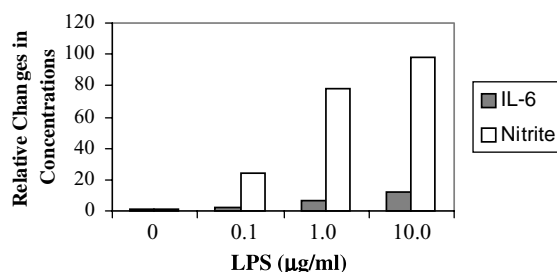


Fig. 3. Accumulation of nitrite and IL-6 in the culture medium of 24 h LPS-stimulated HTC cells.

production of MMP-2 into culture medium as indicated by a gelatin zymograph assay which was not done by either ionomycin or zymosan. LPS, was a mildly active inducer of MMP secretion (Fig. 5). The MW of gelatinolytic bands corresponded to ~17 and 62 kDa.

3.5. RT-PCR analyses for IFN- γ , IL-1, IL-6, iNOS, MMP-2, TGF- β 4, and β -actin transcripts

The HTC cells expressed IFN- γ , IL-1, IL-6, iNOS, TGF- β 4, MMP-2, and β -actin mRNA transcripts

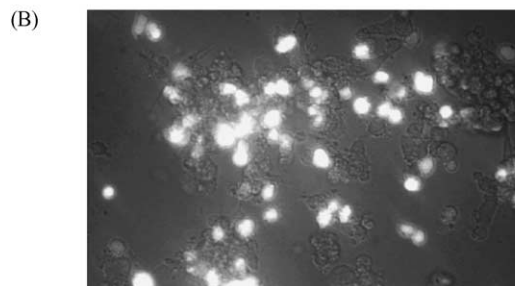
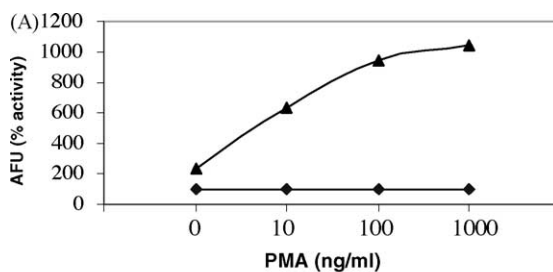


Fig. 4. Stimulation of respiratory burst activities of HTC cells (A) changes in fluorescence intensity of DCF-DA oxidation product at 60 min after stimulation with different concentrations of PMA: (▲) with cells, (◆) without cells. (B) visualization of fluorescence activity of HTC cells cultured on a cover slip after 10 min of stimulation with 100 ng of PMA/ml culture medium.

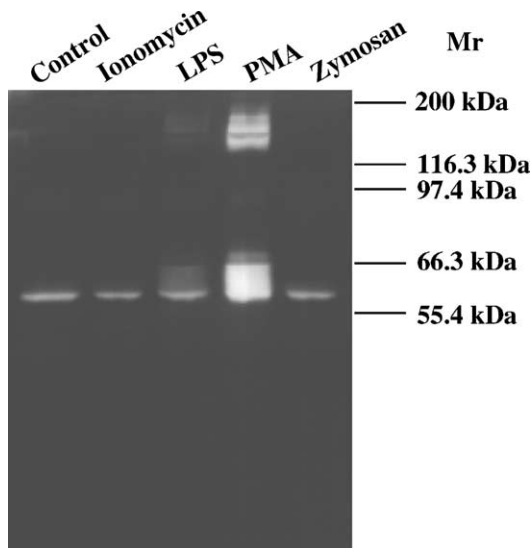


Fig. 5. Gelatin-zymographic analysis of MMP activities of conditioned media of HTC cell cultured with different effectors for 24 h.

(Fig. 6A). A typical electropherogram of the analysis of amplicons with CE-LIF is shown using combined β -actin and MMP-2 PCR products (Fig. 6B). Relative levels of IFN- γ , IL-1, and iNOS calculated with respect to β -actin were upregulated by treatment with LPS. Also the 71 bp size of IL-6 mRNA, which corresponds to the coding region sequence deduced from published IL-6 sequence by Schneider et al. (2001), was up regulated with LPS treatment whereas the MMP-2 mRNA appeared to be reduced, and the levels of TGF- β 4 remained unchanged (Table 4). Analyses using electrophoretic mobility of amplicons on 1.5% agarose gel electrophoresis (Fig. 6A) and electropherogram using capillary electrophoresis followed by LIF detection matched the predictive size of the amplicons.

3.6. Presence of transforming viruses

We screened the HTC cell DNA, along with DNA from some known cell lines for the presence of tumor virus sequences to determine the nature of the immortalization event(s) in HTC cells. Using primers specific for ALV and reticuloendotheliosis proviral LTRs, Marek's disease virus serotype 1 (MDV-1), and vaccine virus herpes virus of turkeys (HVT), and the adventitious agent chicken infectious anemia virus (CIAV) and PCR, our results show that HTC cells, along with few other control lines (DT40, RP9, and

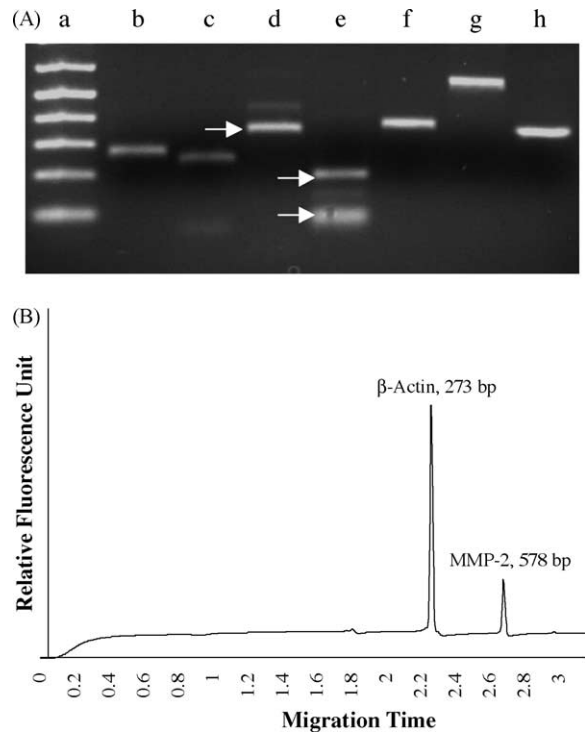


Fig. 6. (A) A typical agarose gel profiles of RT-PCR products of 6 h LPS-stimulated HTC cells using primers against different genes: (a) 1 kb ladder, (b) β -actin (273 bp), (c) interferon- γ (247 bp), (d) interleukin-1 (350 bp, arrow), (e) interleukin-6 (191 bp, upper arrow; 71 bp, lower arrow), (f) inducible nitric oxide synthase (371 bp), (g) matrix metalloproteinase-2 (578 bp), and (h) transforming growth factor- β 4 (340 bp). (B) A typical electropherogram containing equivalent amount of diluted PCR products of β -actin and MMP-2.

Table 4

Changes in the expression of selected genes in HTC cells by LPS treatment. RT-PCR amplified cDNA were analyzed by CE-LIF and the relative mRNA expression was calculated from the peak height ratios of amplicons of individual genes relative to β -actin

Test genes	Relative mRNA expression 0 h LPS treatment	Relative mRNA expression 6 h LPS treatment
IFN- γ	0.001	0.016
IL-1 β	0.189	0.684
IL-6 (191 bp) (71 bp)	0.164, BD	0.111, 0.074
iNOS	0.509	0.729
MMP-2	0.512	0.357
TGF- β 4	0.561	0.577

BDb: Below detection.

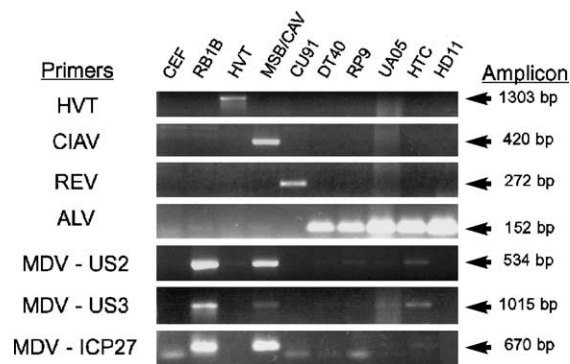


Fig. 7. PCR-based analysis of HTC cell DNA. The HTC cell line was examined for the presence of tumor viruses (ALV, MDV, REV) and other adventitious agents (CAIV and HVT) using the PCR and agent-specific primers (see Table 3). The DNA templates were purified from: specific-pathogen-free, SCWL chicken embryo fibroblasts (CEF), Marek's disease virus (MDV), strain RB1B-infected CEF (RB1B), HVT-infected CEF, MSB-1 cells infected with CAIV-1, ALV-A transformed B cell line (DT40), ALV-B-transformed B cell line (RAV), ALV-5 transformed monocytic line LSCC UA05, HTC and chicken macrophage cell line and amplified.

HD11) were positive for the presence of exogenous ALV LTR sequences. Additionally, the HTC cells were weakly positive for MDV-1 (Fig. 7). HTC DNA was negative for adventitious viruses such as CAIV and vaccine virus HVT, and REV.

3.7. P27 ELISA and the presence of ALV-J in the HTC cells

An ELISA measurement of viral titer using ProFlok kit showed an average calculated value for P27 in cell supernatant to be ≥ 6 ng/ml and in sonicate ≥ 10 ng/ml indicating that the cells were shedding ALV antigens. Further experiments to identify whether the ALV belonged to the 'J' subtype using an indirect immunofluorescence assay failed to show the presence of ALV-J either in HTC cells or in chicken embryo fibroblasts that had been cultured with HTC cell conditioned medium (not shown).

4. Discussion

We report the establishment and characterization of a chicken monocyte cell line HTC which displays many of the characteristics that may be ascribed to

macrophages. While the production of leukocyte acid phosphatase, expression of both class I and class II MHC antigens, hyaluronan binding CD44, the cell surface antigens K1, and K55 (Chung et al., 1991; Cloud et al., 1992; Kaspers et al., 1993) reveal that the cells belong to leukocyte lineage, their abilities to engulf latex particles, and respond to LPS by the production of nitrite and IL-6, and reactive oxygen in response to PMA provide additional functional evidence that these cells belong to myeloid/macrophage lineage. Using several gene-specific primers and RT-PCR the results show that HTC cells constitutively produce IL-1, MMP-2, iNOS, and TGF- β 4 mRNA transcripts and on stimulation with LPS the production of mRNA transcripts for IL-1, NOS was increased but a distinct stimulation of both IFN- γ , and IL-6 transcripts was evident within 6 h. There was no change in TGF- β mRNA and a down regulation of MMP-2 mRNA level was evident on LPS treatment. Although a constitutive presence of NOS mRNA was observed, it is intriguing that there was very little, if any, nitrite present in the unstimulated conditioned medium. The induction of IL-6 mRNA in LPS stimulated HTC cells is at par with bioassay data but the induction of IFN- γ again appears to be intriguing since it is not referred to be common product of macrophages (<http://www.copewithcytokines.de>) although it is known to modulate macrophage function (Lowenthal et al., 2000). However, some recent data in mammalian species suggest that many cells involved in the innate immune response including macrophages possibly produce similar type of cytokines including IFN- γ (Kirby et al., 2002). Also, if IFN- γ mRNA induction by HTC cells is an aberrance related to transformation remains to be seen although there are precedence of many virally transformed cells being capable of producing viral homologs of different cytokines and their receptors (Alcami and Smith, 1995; Nicholas et al., 1997; Parcells et al., 2001). Xing and Schat (2000) have shown that MDV infected fibroblasts also spontaneously produce IFN- γ .

To understand the nature of the immortalization event, we used several avian virus specific primers to identify the viral DNA sequences by PCR amplification. Our results showed the presence of an exogenous ALV and MDV although the MDV genome copy number was low. With the prevalence of ALV in many chicken lines (Payne, 1998; Witter, 1997), it is quite possible that

ALV may have been originally responsible for the transformation. There are two other avian macrophage cell lines, HD11 and MQ-NCSU that are transformed by avian myeloblastosis viruses (Beug et al., 1979; Qureshi et al., 2000). The presence of ALV was evident using a ELISA for P27, an ALV group specific antigen (gag) in the cell culture conditioned medium and HTC cell lysates. P27 gag is a common antigen of all subgroups of ALV which can be present in the blood or cell culture conditioned medium when the virus is being shed actively. However, antibody staining subsequently, suggested that the ALV does not belong to the subgroup J that is known to affect many broiler breeder flocks (Payne, 1998). There are two possibilities for the occurrence of MDV in HTC cells, first, because of opportunistic co-infection of cultures by MDV or through vaccination against MDV which the broilers are subjected to routinely. Retrospectively, another potential mechanism of transformation can be inferred for the presence of both ALV and MDV in the HTC cells. It is known that one or more viruses can play a helper role in the process of cell transformation and MDV is known to augment ALV induced leukosis (Bacon et al., 1989; Pulaski et al., 1992) and herpes virus-induced oncogenesis is enhanced by co-carcinogenic events (de The, 1978). Because, the culture was inadvertently left undisturbed over a period of 25 days and our laboratory is not involved in MDV infection studies, we hypothesize that vaccination or flock exposure or challenge was the origin of MDV sequences in the HTC cells. It is possible that factors derived from both viruses could have contributed to the transformation of HTC cells.

In conclusion, our results indicate that HTC cells belong to a myeloid/macrophage lineage and are capable of growing in medium containing 2% CS both at 37 and 41 °C. They can grow as adherent cells in subconfluent densities. Because of some of these characteristics, we believe that HTC cells may afford a good model to study diverse aspects of cellular regulation and xenobiotic interactions to understand the macrophage function in poultry.

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